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(54) Title: METHOD OF PREPARING A HEAT-TREATED PRODUCT

(57) Abstract: The formation of acrylamide during heat treatment in the production of a food product is reduced by treating the raw material with an enzyme before the heat treatment. The enzyme is capable of reacting on asparagine or glutamine (optionally substituted) as a substrate or is a laccase or a peroxidase.

METHOD OF PREPARING A HEAT-TREATED PRODUCT

FIELD OF THE INVENTION

The present invention relates to a method of preparing a heat-treated product with a low water content from raw material comprising carbohydrate, protein and water. It also relates
5 to an asparaginase for use in the method

BACKGROUND OF THE INVENTION

E. Tabeke et al. (*J. Agric. Food Chem.*, 2002, 50, 4998-5006) reported that acrylamide is formed during heating of starch-rich foods to high temperatures. The acrylamide formation has been ascribed to the Maillard reaction (D.S. Mottram et al., R.H. Stadler et al., *Nature*,
10 419, 3 October 2002, 448-449).

WO 00/56762 discloses expressed sequence tags (EST) from *A. oryzae*.

Kim, K.-W.; Kamerud, J.Q.; Livingston, D.M.; Roon, R.J., (1988) Asparaginase II of *Saccharomyces cerevisiae*. Characterization of the ASP3 gene. *J. Biol. Chem.* 263:11948, discloses the peptide sequence of an extra-cellular asparaginase

15 SUMMARY OF THE INVENTION

According to the invention, the formation of acrylamide during heat treatment of raw material comprising carbohydrate, protein and water is reduced by treating the raw material with an enzyme before the heat treatment. Accordingly, the invention provides a method of preparing a heat-treated product, comprising the sequential steps of:

- 20 a) providing a raw material which comprises carbohydrate, protein and water
 b) treating the raw material with an enzyme, and
 c) heat treating to reach a final water content below 35 % by weight.

The enzyme is capable of reacting on asparagine or glutamine (optionally substituted) as a substrate or is a laccase or a peroxidase.

25 The invention also provides an asparaginase for use in the process and a polynucleotide encoding the asparaginase.

DETAILED DESCRIPTION OF THE INVENTION

Raw material and enzyme treatment

The raw material comprises carbohydrate, protein and water, typically in amounts of
30 10-90 % or 20-50 % carbohydrate of the total weight. The carbohydrate may consist mainly of starch, and it may include reducing sugars such as glucose, e.g. added as glucose syrup,

honey or dry dextrose. The protein may include free amino acids such as asparagine and glutamine (optionally substituted).

The raw material may include tubers, potatoes, grains, oats, barley, corn (maize), wheat, nuts, fruits, dried fruit, bananas, sesame, rye and/or rice.

5 The raw material may be in the form of a dough comprising finely divided ingredients (e.g. flour) with water. The enzyme treatment may be done by mixing (kneading) the enzyme into the dough and optionally holding to let the enzyme act. The enzyme may be added in the form of an aqueous solution, a powder, a granulate or agglomerated powder. The dough may be formed into desired shapes, e.g. by sheeting, cutting and/or extrusion.

10 The raw material may also be in the form of intact vegetable pieces, e.g. slices or other pieces of potato, fruit or bananas, whole nuts, whole grains etc. The enzyme treatment may comprise immersing the vegetable pieces in an aqueous enzyme solution and optionally applying vacuum infusion. The intact pieces may optionally be blanched by immersion in hot water, e.g. at 70-100°C, either before or after the enzyme treatment.

15 The raw material may be grain intended for malting, e.g. malting barley or wheat. The enzyme treatment of the grain may be done before, during or after the malting (germination).

The raw material before heat treatment typically has a water content of 10-90 % by weight and is typically weakly acidic, e.g. having a pH of 5-7.

Heat treatment

20 The process of the invention involves a heat treatment at high temperature to reach a final water content (moisture content) in the product below 35 % by weight, typically 1-20 %, 1-10 % or 2-5 %. During the heat treatment, the temperature at the surface of the product may reach 110-220°C, e.g. 110-170°C or 120-160°C.

25 The heat treatment may involve, frying, particularly deep frying in tri- and/or di-glycerides (animal or vegetable oil or fat, optionally hydrogenated), e.g. at temperatures of 150-180°C. The heat treatment may also involve baking in hot air, e.g. at 160-310°C or 200-250°C for 2-10 minutes, or hot-plate heating. Further, the heat treatment may involve kilning of green malt.

Heat-treated product

30 The process of the invention may be used to produce a heat-treated product with low water content from raw material containing carbohydrate and protein, typically starchy food products fried or baked at high temperatures. The heat-treated product may be consumed directly as an edible product or may be used as an ingredient for further processing to prepare an edible or potable product.

Examples of products to be consumed directly are potato products, potato chips (crisps), French fries, hash browns, roast potatoes, breakfast cereals, crisp bread, muesli, biscuits, crackers, snack products, tortilla chips, roasted nuts, rice crackers (Japanese "senbei"), wafers, waffles, hot cakes, and pancakes.

- 5 Malt (e.g. caramelized malt or so-called chocolate malt) is generally further processed by mashing and brewing to make beer.

Enzyme capable of reacting with asparagine or glutamine (optionally substituted) as a substrate

- The enzyme may be capable of reacting with asparagine or glutamine which is optionally glycosylated or substituted with a peptide at the alpha-amino and/or the carboxyl position. The enzyme may be an asparaginase, a glutaminase, an L-amino acid oxidase, a glycosylasparaginase, a glycoamidase or a peptidoglutaminase.

- The glutaminase (EC 3.5.1.2) may be derived from *Escherichia coli*. The L-amino acid oxidase (EC 1.4.3.2) capable of reacting with asparagine or glutamine (optionally glycosylated) as a substrate may be derived from *Trichoderma harzianum* (WO 94/25574). The glycosylasparaginase (EC 3.5.1.26, aspartylglucosaminidase, N4-(N-acetyl-beta-glucosaminyl)-L-asparagine amidase) may be derived from *Flavobacterium meningosepticum*. The glycoamidase (peptide N-glycosidase, EC 3.5.1.52) may be derived from *Flavobacterium meningosepticum*. The peptidoglutaminase may be peptidoglutaminase I or II (EC 3.5.1.43, EC 3.5.1.44).

- 20 The enzyme is used in an amount which is effective to reduce the amount of acrylamide in the final product. The amount may be in the range 0.1-100 mg enzyme protein per kg dry matter, particularly 1-10 mg/kg. Asparaginase may be added in an amount of 10-100 units per kg dry matter where one unit will liberate 1 micromole of ammonia from L-asparagine per min at pH 8.6 at 37 °C

25 Asparaginase

- The asparaginase (EC 3.5.1.1) may be derived from *Saccharomyces cerevisiae*, *Candida utilis*, *Escherichia coli*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Fusarium graminearum*, or *Penicillium citrinum*. It may have the amino acid sequence shown in SEQ ID NO: 2 (optionally truncated to residues 27-378, 30-378, 75-378 or 80-378), 4, 6, 8, 10, 12 or 13 or a sequence which is at least 90 % (particularly at least 95 %) identical to one of these. It may be produced by use of the genetic information in SEQ ID NO: 1, 3, 5, 7, 9 or 11, e.g., as described in an example.

- Whitehead Institute, MIT Center for Genome Research, Fungal Genome Initiative has published *A. nidulans* release 1 and *F. graminearum* release 1 on the Internet at <http://www-genome.wi.mit.edu/ftp/distribution/annotation/> under the *Aspergillus* Sequencing Project and

the *Fusarium graminearum* Sequencing Project. Preliminary sequence data for *Aspergillus fumigatus* was published on The Institute for Genomic Research website at <http://www-genome.wi.mit.edu/ftp/distribution/annotation/>.

The inventors inserted the gene encoding the asparaginase from *A. oryzae* into *E. coli* and deposited the clone under the terms of the Budapest Treaty with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig. The deposit number was DSM 15960, deposited on 6 October 2003.

Alignment and Identity

The enzyme and the nucleotide sequence of the invention may have homologies to the disclosed sequences of at least 90 % or at least 95 %, e.g. at least 98 %.

For purposes of the present invention, alignments of sequences and calculation of identity scores were done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Laccase or peroxidase

The laccase (EC 1.10.3.2) may be of plant or microbial origin, e.g. from bacteria or fungi (including filamentous fungi and yeasts). Examples include laccase from *Aspergillus*, *Neurospora*, e.g., *N. crassa*, *Podospira*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, e.g., *T. villosa* and *T. versicolor*, *Rhizoctonia*, e.g., *R. solani*, *Coprinus*, e.g., *C. cinereus*, *C. comatus*, *C. friesii*, and *C. plicatilis*, *Psathyrella*, e.g., *P. condelleana*, *Panaeolus*, e.g., *P. papilionaceus*, *Myceliophthora*, e.g., *M. thermophila*, *Schytalidium*, e.g., *S. thermophilum*, *Polyporus*, e.g., *P. pinsitus*, *Phlebia*, e.g., *P. radita*, or *Coriolus*, e.g., *C. hirsutus*.

The peroxidase (EC 1.11.1.7) may be from plants (e.g. horseradish or soybean peroxidase) or microorganisms such as fungi or bacteria, e.g. *Coprinus*, in particular *Coprinus cinereus* f. *microsporus* (IFO 8371), or *Coprinus macrorrhizus*, *Pseudomonas*, e.g. *P. fluorescens* (NRRL B-11), *Streptovorticillium*, e.g. *S. verticillium* ssp. *verticillium* (IFO 13864), *Streptomyces*, e.g. *S. thermoviolaceus* (CBS 278.66), *Streptomyces*, e.g. *S. viridosporus* (ATCC 39115), *S. badius* (ATCC 39117), *S. phaeochromogenes* (NRRL B-3559), *Pseudomonas*, e.g. *P. pyrocinia* (ATCC 15958), *Fusarium*, e.g. *F. oxysporum* (DSM 2672) and *Bacillus*, e.g. *B. stearothermophilus* (ATCC 12978).

Oxidoreductase capable of reacting with a reducing sugar as a substrate

The method of the invention may comprise treating the raw material with an oxidoreductase capable of reacting with a reducing sugar as a substrate. The oxidoreductase may be an oxidase or dehydrogenase capable of reacting with a reducing sugar as a substrate such as
 5 glucose and maltose.

The oxidase may be a glucose oxidase, a pyranose oxidase, a hexose oxidase, a galactose oxidase (EC 1.1.3.9) or a carbohydrate oxidase which has a higher activity on maltose than on glucose. The glucose oxidase (EC 1.1.3.4) may be derived from *Aspergillus niger* e.g. having the amino acid sequence described in US 5094951. The hexose oxidase (EC 1.1.3.5)
 10 may be derived from algal species such as *Iridophycus flaccidum*, *Chondrus crispus* and *Euthora cristata*. The pyranose oxidase may be derived from *Basidiomycete* fungi, *Peniophora gigantea*, *Aphylllophorales*, *Phanerochaete chrysosporium*, *Polyporus pinsitus*, *Bierkandera adusta* or *Phlebiopsis gigantea*. The carbohydrate oxidase which has a higher activity on maltose than on glucose may be derived from *Microdochium* or *Acremonium*, e.g. from *M. nivale*
 15 (US 6165761), *A. strictum*, *A. fusidioides* or *A. potronii*.

The dehydrogenase may be glucose dehydrogenase (EC 1.1.1.47, EC 1.1.99.10), galactose dehydrogenase (EC 1.1.1.48), D-aldohexose dehydrogenase (EC 1.1.1.118, EC 1.1.1.119), cellobiose dehydrogenase (EC 1.1.5.1, e.g. from *Humicola insolens*), fructose dehydrogenase (EC 1.1.99.11, EC 1.1.1.124, EC 1.1.99.11), aldehyde dehydrogenase (EC
 20 1.2.1.3, EC 1.2.1.4, EC 1.2.1.5). Another example is glucose-fructose oxidoreductase (EC 1.1.99.28).

The oxidoreductase is used in an amount which is effective to reduce the amount of acrylamide in the final product. For glucose oxidase, the amount may be in the range 50-20,000 (e.g. 100-10,000 or 1,000-5,000) GODU/kg dry matter in the raw material. One GODU
 25 is the amount of enzyme which forms 1 μ mol of hydrogen peroxide per minute at 30°C, pH 5.6 (acetate buffer) with glucose 16.2 g/l (90 mM) as substrate using 20 min. incubation time. For other enzymes, the dosage may be found similarly by analyzing with the appropriate substrate.

EXAMPLES**Media**30 **DAP2C-1**11g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1g KH_2PO_4

2g Citric acid, monohydrate

30g maltodextrin

6g $K_3PO_4 \cdot 3H_2O$

0.5g yeast extract

0.5ml trace metals solution

1ml Pluronic PE 6100 (BASF, Ludwigshafen, Germany)

- 5 Components are blended in one liter distilled water and portioned out to flasks, adding 250 mg $CaCO_3$ to each 150ml portion.

The medium is sterilized in an autoclave. After cooling the following is added to 1 liter of medium:

- 23 ml 50% w/v $(NH_4)_2HPO_4$, filter sterilized
10 33 ml 20% lactic acid, filter sterilized

Trace metals solution

- 6.8g $ZnCl_2$
2.5g $CuSO_4 \cdot 5H_2O$
0.24g $NiCl_2 \cdot 6H_2O$
15 13.9g $FeSO_4 \cdot 7H_2O$
8.45g $MnSO_4 \cdot H_2O$
3g Citric acid, monohydrate
Components are blended in one liter distilled water.

Asparaginase activity assay

20 *Stock solutions*

- 50 mM Tris buffer, pH 8.6
189mM L-Asparagine solution
1.5 M Trichloroacetic Acid (TCA)
Nessler's reagent, Aldrich Stock No. 34,514-8 (Sigma-Aldrich, St. Louis, Mo. USA)
25 Asparaginase, Sigma Stock No. A4887 (Sigma-Aldrich, St. Louis, Mo. USA)

Assay

Enzyme reaction:

- 500 micro-l buffer
100 micro-l L-asparagine solution
30 350 micro-l water
are mixed and equilibrated to 37 °C.
100 micro-l of enzyme solution is added and the reactions are incubated at 37 °C for 30 minutes.

The reactions are stopped by placing on ice and adding 50 micro-l of 1.5M TCA.

The samples are mixed and centrifuged for 2 minutes at 20,000 g

Measurement of free ammonium:

50 micro-l of the enzyme reaction is mixed with 100 micro-l of water and 50 micro-l of Nessler's reagent. The reaction is mixed and absorbance at 436nm is measured after 1 minute.

Standard:

The asparaginase stock (Sigma A4887) is diluted 0.2, 0.5, 1, 1.5, 2, and 2.5 U/ml.

Example 1: Expression of an asparaginase from *Aspergillus oryzae* in *Aspergillus oryzae*

10 Libraries of cDNA of mRNA from *Aspergillus oryzae* were generated, sequenced and stored in a computer database as described in WO 00/56762.

The peptide sequence of asparaginase II from *Saccharomyces cerevisiae* (Kim, K.-W.; Kamerud, J.Q.; Livingston, D.M.; Roon, R.J., (1988) *Asparaginase II of Saccharomyces cerevisiae*. Characterization of the ASP3 gene. *J. Biol. Chem.* 263:11948), was compared to translations of the *Aspergillus oryzae* partial cDNA sequences using the TFASTXY program, version 3.2107 (Pearson et al, *Genomics* (1997) 46:24-36). One translated *A. oryzae* sequence was identified as having 52% identity to yeast asparaginase II through a 165 amino acid overlap. The complete sequence of the cDNA insert of the corresponding clone (deposited as DSM 15960) was determined and is presented as SEQ ID NO: 1, and the peptide translated from this sequence, AoASP, is presented as SEQ ID NO: 2. This sequence was used to design primers for PCR amplification of the AoASP encoding-gene from DSM 15960, with appropriate restriction sites added to the primer ends to facilitate sub-cloning of the PCR product (primers AoASP7 and AoASP8, SEQ ID NOS: 14 and 15). PCR amplification was performed using Extensor Hi-Fidelity PCR Master Mix (ABgene, Surrey, U.K.) following the manufacturer's instructions and using an annealing temperature of 55°C for the first 5 cycles and 65°C for an additional 30 cycles and an extension time of 1.5 minutes.

The PCR fragment was restricted with *Bam*HI and *Hind*III and cloned into the *Aspergillus* expression vector pMStr57 using standard techniques. The expression vector pMStr57 contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to the *Aspergillus* NA2 promoter as described for the vector pMT2188 in WO 01/12794, and has sequences for selection and propagation in *E. coli*, and selection and expression in *Aspergillus*. Specifically, selection in *Aspergillus* is facilitated by the *amdS* gene of *Aspergillus nidulans*, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (*tpi*) encoding-gene from

Aspergillus nidulans, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The asparaginase-encoding gene of the resulting *Aspergillus* expression construct, pMStr90, was sequenced and the sequence agreed completely with that determined previously for the insert of DSM 15960

- 5 The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr90 using standard techniques (Christensen, T. et al., (1988), Biotechnology 6, 1419-1422). Transformants were cultured in DAP2C-1 medium shaken at 200 RPM at 30°C and expression of AoASP was monitored by SDS-PAGE and by measuring enzyme activity.

Example 2: Purification of Asparaginase

- 10 Culture broth from the preceding example was centrifuged (20000 x g, 20 min) and the supernatants were carefully decanted from the precipitates. The combined supernatants were filtered through a Seitz EKS plate in order to remove the rest of the *Aspergillus* host cells. The EKS filtrate was transferred to 10 mM Tris/HCl, pH 8 on a G25 sephadex column and applied to a Q sepharose HP column equilibrated in the same buffer. After washing the Q sepharose HP column extensively with the equilibration buffer, the asparaginase was eluted with a
15 linear NaCl gradient (0 → 0.5M) in the same buffer. Fractions from the column were analysed for asparaginase activity (using the pH 6.0 Universal buffer) and fractions with activity were pooled. Ammonium sulfate was added to the pool to 2.0M final concentration and the pool was applied to a Phenyl Toyopearl S column equilibrated in 20 mM succinic acid, 2.0M (NH₄)₂SO₄,
20 pH 6.0. After washing the Phenyl column extensively with the equilibration buffer, the enzyme was eluted with a linear (NH₄)₂SO₄ gradient (2.0 → 0M) in the same buffer. Fractions from the column were again analysed for asparaginase activity and active fractions were further analysed by SDS-PAGE. Fractions, which was judged only to contain the asparaginase, were pooled as the purified preparation and was used for further characterization. The purified as-
25 paraginase was heterogeneously glycosylated judged from the coomassie stained SDS-PAGE gel and in addition N-terminal sequencing of the preparation revealed that the preparation contained different asparaginase forms, as four different N-termini were found starting at amino acids A₂₇, S₃₀, G₇₅ and A₈₀ respectively of SEQ ID NO: 2. However, the N-terminal sequencing also indicated that the purified preparation was relatively pure as no other N-terminal se-
30 quences were found by the analysis.

Example 3: Properties of asparaginase

The purified asparaginase from the preceding example was used for characterization.

Asparaginase assay

- A coupled enzyme assay was used. Asparaginase was incubated with asparagine
35 and the liberated ammonia was determined with an Ammonia kit from Boehringer Mannheim

(cat. no. 1 112 732) based on glutamate dehydrogenase and NADH oxidation to NAD⁺ (can be measured as a decrease in A_{375}). Hence the decrease in absorbance at 375 nm was taken as a measure of asparaginase activity.

Asparagine substrate :	10mg/ml L-asparagine (Sigma A-7094) was dissolved in Universal buffers and pH was adjusted to the indicated pH-values with HCl or NaOH.
Temperature :	controlled
Universal buffers :	100 mM succinic acid, 100 mM HEPES, 100 mM CHES, 100 mM CABS, 1 mM CaCl ₂ , 150 mM KCl, 0.01% Triton X-100 adjusted to pH-values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with HCl or NaOH.
Stop reagent :	500 mM TCA (Trichloroacetic acid).
Assay buffer :	1.0M KH ₂ PO ₄ /NaOH, pH 7.5.
Ammonia reagent A :	1 NADH tablet + 1.0 ml Bottle 1 (contain 2-oxoglutarate (second substrate) and buffer) + 2.0 ml Assay buffer.
Ammonia reagent B :	40 micro-l Bottle 3 (contain glutamate dehydrogenase) + 1460 micro-l Assay buffer.

5 450 micro-l asparagine substrate was placed on ice in an Eppendorf tube. 50 micro-l asparaginase sample (diluted in 0.01% Triton X-100) was added. The assay was initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate (1400 rpm). The incubation was stopped by transferring the tube back to
10 the ice bath and adding 500 micro-l Stop reagent. The tube was vortexed and centrifuged shortly in an icecold centrifuge to precipitate the proteins in the tube. The amount of ammonia liberated by the enzyme was measured by the following procedure: 20 micro-l supernatant was transferred to a microtiter plate, 200 micro-l Ammonia reagent A was added and A_{375} was read ($A_{375}(\text{initial})$). Then 50 micro-l Ammonia reagent B was added and after 10 minutes at room
15 temperature the plate was read again ($A_{375}(\text{final})$). $A_{375}(\text{initial}) - A_{375}(\text{final})$ was a measure of asparaginase activity. A buffer blind was included in the assay (instead of enzyme) and the decrease in A_{375} in the buffer blind was subtracted from the enzyme samples.

pH-activity, pH-stability, and temperature-activity of asparaginase

The above asparaginase assay was used for obtaining the pH-activity profile, the pH-
20 stability profile as well as the temperature-activity profile at pH 7.0. For the pH-stability profile the asparaginase was diluted 7x in the Universal buffers and incubated for 2 hours at 37°C.

After incubation the asparaginase samples were transferred to neutral pH, before assay for residual activity, by dilution in the pH 7 Universal buffer.

The results for the: pH-activity profile at 37°C were as follows, relative to the residual activity at after 2 hours at pH 7.0 and 5°C :

pH	Asparaginase
2	0.00
3	0.01
4	0.10
5	0.53
6	0.95
7	1.00
8	0.66
9	0.22
10	0.08
11	0.00

5

The results for the pH-stability profile (residual activity after 2 hours at 37°C) were as follows:

pH	Asparaginase
2.0	0.00
3.0	0.00
4.0	1.06
5.0	1.08
6.0	1.09
7.0	1.09
8.0	0.92
9.0	0.00
10.0	0.00
11.0	0.00
12.0	0.00
	1.00

The results for the temperature activity profile (at pH 7.0) were as follows:

Temp (°C)	Asparaginase
15	0.24
25	0.39
37	0.60
50	0.81
60	1.00
70	0.18

Other characteristics

The relative molecular weight as determined by SDS-PAGE was seen as a broad band (a smear) at M_r = 40-65 kDa.

N-terminal sequencing showed four different terminals, corresponding to residues 27-37, 30-40, 75-85 and 80-91 of SEQ ID NO: 2, respectively.

Example 3: Cloning of asparaginase from *Penicillium citrinum*

Penicillium citrinum was grown in MEX-1 medium (Medium B in WO 98/38288) in flasks shaken at 150RPM at 26°C for 3 and 4 days. Mycelium was harvested, a cDNA library constructed, and cDNAs encoding secreted peptides were selected and sequenced by the methods described in WO 03/044049. Comparison to known sequences by methods described in WO 03/044049 indicated that *Penicillium* sequence ZY132299 encoded an asparaginase. The complete sequence of the corresponding cDNA was determined and is presented as SEQ ID NO: 11, and the peptide translated from this sequence is presented as SEQ ID NO: 12.

Example 4: Effect of asparaginase on acrylamide content in potato chips

Asparaginase from *A. oryzae* having the amino acid sequence shown in SEQ ID NO: 2 was prepared and purified as in Examples 1-2 and added at various dosages to potato chips made from 40 g of water, 52.2 g of dehydrated potato flakes, 5.8 g of potato starch and 2 g of salt.

The flour and dry ingredients were mixed for 30 sec. The salt and enzyme were dissolved in the water, and the solution was adjusted to 30°C. The solution was added to the flour. The dough was further mixed for 15 min. The mixed dough was placed in a closed plastic bag and allowed to rest for 15 min at room temperature.

The dough was then initially compressed for 60 sec in a dough press.

The dough was sheeted and folded in a noodle roller machine until an approx. 5-10 mm dough is obtained. The dough was then rolled around a rolling pin and allowed to rest for

30 min in a plastic bag at room temperature. The dough was sheeted further to a final sheet thickness of approx 1.2 mm.

The sheet was cut into squares of approx 3 x 5 cm.

The sheets were placed in a frying basket, placed in an oil bath and fried for 45 sec at 180° C. The noodle basket was held at a 45° angle until the oil stopped dripping. The products were removed from the basket and left to cool on dry absorbent paper.

The potato chips were homogenized and analyzed for acrylamide. The results were as follows:

Asparaginase dosage U/kg potato dry matter	Acrylamide Micro-g per kg
0	5,200
100	4,600
500	3,100
1000	1,200
2000	150

The results demonstrate that the asparaginase treatment is effective to reduce the acrylamide content in potato chips, that the acrylamide reduction is clearly dosage dependent, and that the acrylamide content can be reduced to a very low level.

Example 5: Effect of various enzymes on acrylamide content in potato chips

Potato chips were made as follows with addition of enzyme systems which are capable of reacting on asparagine, as indicated below.

Recipe:

Tap water	40 g
Potato flakes dehydrated	52.2 g
Potato starch	5.8 g
Salt	2 g

Dough Procedure:

The potato flakes and potato starch are mixed for 30 sec in a mixer at speed 5. Salt and enzyme are dissolved in the water. The solution is adjusted to 30°C +/- 1°C. Stop mixer, add all of the salt/enzyme solution to flour. The dough is further mixed for 15 min.

Place mixed dough in plastic bag, close bag and allow the dough to rest for 15 min at room temperature.

The dough is then initially compressed for 60 sec in a dough press.

The dough is sheeted and folded in a noodle roller machine until an approx. 5-10 mm dough is obtained. The dough is then rolled around a rolling pin and the dough is allowed to rest for 30 min in a plastic bag at room temperature. The dough is sheeted further to a final sheet thickness of approx 1.2 mm.

- 5 Cut the sheet into squares of approx 3 x 5 cm.

Sheets are placed in a frying basket, placed in the oil bath and fried for 60 sec at 180°C. Hold the noodle basket at a 45° angle and let the product drain until oil stops dripping. Remove the products from the basket and leave them to cool on dry absorbent paper.

The results from acrylamide analysis were as follows:

Enzyme	Enzyme dosage per kg of potato dry matter	Acrylamide Micro-g per kg
None (control)	0	4,100
Asparaginase from <i>Erwinia Chrysanthemi</i> A-2925	1000 U/kg	150
Glutaminase (product of Daiwa)	50 mg enzyme protein/kg	1,800
Amino acid oxidase from <i>Trichoderma harzianum</i> described in WO 9425574.	50 mg enzyme protein/kg	1,300
Laccase from <i>Myceliophthora thermophila</i> + peroxidase from <i>Coprinus</i>	5000 LAMU/kg + 75 mg enzyme protein/kg	2,000

10

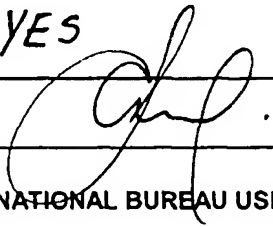
The results demonstrate that all the tested enzyme systems are effective in reducing the acrylamide content of potato chips.

PCT

Original (for SUBMISSION) - printed on 10.10.2003 09:39:26 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.07.2003)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	10347-WO
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	4
1-2	line	5-7
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	06 October 2003 (06.10.2003)
1-3-4	Accession Number	DSMZ 15960
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications	NONE
	These indications will be submitted to the International Bureau later	

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE


DSMZ
Deutsche Sammlung von
Mikroorganismen und
Zellkulturen GmbH



INTERNATIONAL FORM

Novozymes A/S
Krogshøjvej 36
DK-2880 Bagsvaerd

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page


I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Novozymes A/S Krogshøjvej 36 Address: DK-2880 Bagsvaerd	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15960 Date of the deposit or the transfer ¹ : 2003-10-06
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2003-10-06 On that date, the said microorganism was <input checked="" type="checkbox"/> viable <input type="checkbox"/> no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2003-10-13

- ¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
³ Mark with a cross the applicable box.
⁴ Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDUREDSMZ
Deutsche Sammlung von
Mikroorganismen und
Zellkulturen GmbH

INTERNATIONAL FORM

Novozymes A/S
Krogshøjvej 36
DK-2880 BagsvaerdRECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: NN049697	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15960
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: () a scientific description (x) a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2003-10-06 (Date of the original deposit) ¹ .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2003-10-13

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

CLAIMS

1. A method of preparing a heat-treated product, comprising the sequential steps of:
 - a) providing a raw material which comprises carbohydrate, protein and water
 - b) treating the raw material with an enzyme capable of reacting on asparagine or glutamine (optionally substituted) as a substrate, a laccase or a peroxidase, and
 - 5 c) heat treating to reach a final water content below 35 % by weight.
2. The method of the preceding claim wherein the enzyme capable of reacting on asparagine or glutamine (optionally substituted) as a substrate is an asparaginase, a glutaminase, an L-amino acid oxidase, a glycosylasparaginase, a glycoamidase (peptide N-glycosidase) or
10 a peptidoglutaminase.
3. The method of the preceding claim wherein the asparaginase has an amino acid sequence which is at least 90 % identical to SEQ ID NO: 2 (optionally truncated to residues 27-378, 30-378, 75-378 or 80-378), 4, 6, 8, 10, 12 or 13.
4. The method of any preceding claim which further comprises treating the raw material
15 with an oxidoreductase capable of reacting with a reducing sugar as a substrate.
5. The method of the preceding claim wherein the oxidoreductase capable of reacting with a reducing sugar as a substrate is a glucose oxidase, a pyranose oxidase, a hexose oxidase, a galactose oxidase (EC 1.1.3.9) or a carbohydrate oxidase which has a higher activity on maltose than on glucose.
- 20 6. The method of any preceding claim wherein the raw material is in the form of a dough and the enzyme treatment comprises mixing the enzyme into the dough and optionally holding.
7. The method of any preceding claim wherein the raw material comprises intact vegetable pieces and the enzyme treatment comprises immersing the potato pieces in an aqueous solution of the enzyme.
- 25 8. The method of any preceding claim wherein the raw material comprises a potato product.

9. A polypeptide having asparaginase activity and having an amino acid sequence which is at least 90 % identical with SEQ ID NO: 2 (optionally truncated to residues 27-378, 30-378, 75-378 or 80-378) or SEQ ID NO: 12.
10. A polynucleotide encoding the polypeptide of the preceding claim.
- 5 11. A polynucleotide which encodes an asparaginase and which comprises a nucleotide sequence which is at least 90 % identical to the coding sequences of SEQ ID NO: 1 or 11.

10347-WO-ST25
SEQUENCE LISTING

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Page 4

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Val	Tyr	Pro	Arg	Ala	Thr	Ser	Pro	Asn	Ser	Thr	
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Thr	Ile	Leu	Ala	Thr	Gly	Gly	Thr	Ile	Ala	Gly	
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Thr	Ala	Thr	Thr	Gly	Tyr	Thr	Ala	Gly	Ala	Ile	
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Gln	Val	Ala	Asn	Val	Gly	Ser	Pro	Asp	Val	Thr	
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Phe	Phe	Leu	Asp	Ala	Thr	Val	Asn	Cys	Gly	Lys	
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Gly	Ala	Met	Arg	Pro	Ala	Thr	Ala	Ile	Ser	Ala	
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Leu	Leu	Gln	Ala	Val	Thr	Val	Ala	Ala	His	Pro	
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Gly	Ala	Leu	Val	Val	Met	Asn	Asp	Arg	Ile	Val	
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tcc	aag	aca	aac	gcc	aac	acc	atg	gac	acc	ttc	785
Ser	Lys	Thr	Asn	Ala	Asn	Thr	Met	Asp	Thr	Phe	
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Gly	Asn	Leu	Gly	Ala	Ile	Ile	Ser	Asn	Lys	Pro	
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ccc	gtc	atg	ccc	acc	ggt	aag	acc	act	ttc	gac	881
Pro	Val	Met	Pro	Thr	Gly	Lys	Thr	Thr	Phe	Asp	
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 Arg Ser Val Ser Ser Gly Tyr Tyr Asp Ala Ile
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 Asp Asp Ile Ala Ser Thr His Ser Leu Pro Val Val Leu Ser Thr Arg
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acc ggc aac ggc gaa gtc gcc atc aca gac agc gag acc aca att gag 1183
 Thr Gly Asn Gly Glu Val Ala Ile Thr Asp Ser Glu Thr Thr Ile Glu
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Val Ala Asn Val Ala Gly Ile Gln Val Ala Asn Val Gly Ser Pro Asp

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Val Cys Asp Asp Pro Thr Met	Ser Gly Ala Val Ile Thr His Gly Thr	
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Asp Thr Leu Glu Glu Thr Ala Phe Phe Leu	Asp Ala Thr Val Asn Cys	
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Gly Lys Pro Ile Val Val Val Gly Ala Met	Arg Pro Ala Thr Ala Ile	
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Ser Ala Asp Gly Pro Phe Asn Leu Leu Gln Ala Val Thr Val Ala Ala		
	180 185	190
His Pro Thr Ala Arg Asn Arg Gly Ala Leu Val Val Met Asn Asp Arg		
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Ile Val Ser Ala Tyr Tyr Val Ser Lys Thr Asn Ala Asn Thr Met Asp		
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Thr Phe Lys Ala Val Glu Met Gly Asn Leu Gly Ala Ile Ile Ser Asn		
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Lys Pro Tyr Phe Phe Tyr Pro Pro Val Met Pro Thr Gly Lys Thr Thr		
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Phe Asp Val Arg Asn Val Ala Ser Ile Pro Arg Val Asp Ile Leu Tyr		
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Ser Tyr Gln Asp Met Gln Asn Asp Thr Leu Tyr Asp Ala Val Asp Asn		
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Gly Ala Lys Gly Ile Val Val Arg Ser Val Ser Ser Gly Tyr Tyr Asp		
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Ala Ile Asp Asp Ile Ala Ser Thr His Ser Leu Pro Val Val Leu Ser		
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Thr Arg Thr Gly Asn Gly Glu Val Ala Ile Thr Asp Ser Glu Thr Thr		
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Ile Glu Ser Gly Phe Leu Asn Pro Gln Lys Ala Arg Ile Leu Leu Gly		
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Ala	Val	Pro	Gln	Leu	Cys	Asn	Val	Ser	Asn	Val	Arg	Gly	Val	Gln	Ile			
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Asp	Leu	Thr	Val	Gln	Ser	Glu	Lys	Pro	Val	Val	Met	Val	Gly	Ser	Met			
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cgt	cct	gcc	act	gcc	atc	agc	gct	gat	ggg	ccc	atc	aac	ctc	ctg	tct	644		
Arg	Pro	Ala	Thr	Ala	Ile	Ser	Ala	Asp	Gly	Pro	Ile	Asn	Leu	Leu	Ser			
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gct	gtt	cga	ttg	gca	ggg	agc	aag	agt	gcc	aag	ggg	cgc	ggg	aca	atg	692		
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Gly Ala Phe Glu Asn Ile Gln Pro Val Phe Trp Tyr Pro Ala Ser Arg	
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Pro Leu Gly His His Tyr Phe Asn Ile Ser Ala Ser Ser Pro Lys Lys	
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gct ctt cct cag gtt gac gtt ttg tac ggc cac caa gaa gcg gac ccc	932
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Glu Leu Phe Gln Ala Ala Val Asp Ser Gly Ala Gln Gly Ile Val Leu	
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Ala Gly Leu Gly Ala Gly Gly Trp Pro Asp Glu Ala Ala Asp Glu Ile	
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Lys Lys Val Leu Asn Glu Thr Asn Ile Pro Val Val Val Ser Arg Arg	
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Thr Ala Trp Gly Tyr Val Gly Glu Arg Pro Phe Gly Ile Gly Ala Gly	
325 330 335 340	
tac ttg aac cct tcc aag gcc aga atc caa ctg caa ctt gcg ctt gag	1172
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Asn Ile Thr Ile Phe Ala Thr Gly Gly Thr Ile Ala Gly Ser Ala Gly
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 115 120 125
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 Asn Leu Leu Ser Ala Val Arg Leu Ala Gly Ser Lys Ser Ala Lys Gly
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 Thr Val Lys Ser His Ala Asn Ala Val Gln Thr Phe Ile Ala Glu Asp
 210 215 220
 Gln Gly Tyr Leu Gly Ala Phe Glu Asn Ile Gln Pro Val Phe Trp Tyr
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 Pro Ala Ser Arg Pro Leu Gly His His Tyr Phe Asn Ile Ser Ala Ser
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 Ser Pro Lys Lys Ala Leu Pro Gln Val Asp Val Leu Tyr Gly His Gln
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 Glu Ala Asp Pro Glu Leu Phe Gln Ala Ala Val Asp Ser Gly Ala Gln
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 290 295 300
 Ala Asp Glu Ile Lys Lys Val Leu Asn Glu Thr Asn Ile Pro Val Val
 305 310 315 320
 Val Ser Arg Arg Thr Ala Trp Gly Tyr Val Gly Glu Arg Pro Phe Gly
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 Met Val Ala Ala Ala Pro Ser Ile Cys Ser Gly Pro Ala Ala Ser Ser
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 Thr Ile Lys Met Ala Ser Ser Ala Ser Trp Thr Thr Tyr Leu Trp
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 cgg ctt atc cta gct gtg ctg gct cct tca acg gcc ctg ctg cct ttt 256
 Arg Leu Ile Leu Ala Val Leu Ala Pro Ser Thr Ala Leu Leu Pro Phe
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 Gly Ala Trp Val Val Ser Val Trp Gly Ser Pro Val Leu Asp Leu His
 65 70 75
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 Ile Pro Phe Glu Ile Ser Thr Thr Ser Gly Phe Asn Cys Phe Asn Pro
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 Asn Leu Pro Asn Val Thr Ile Tyr Ala Thr Gly Gly Thr Ile Ala Gly
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 tcc gca agc tcg gct gat cag acc acg gga tac cgg tca gct gcg tta 496
 Ser Ala Ser Ser Ala Asp Gln Thr Thr Gly Tyr Arg Ser Ala Ala Leu
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Ser	Pro	Phe	Thr	Gln	Gly	Ala	Val	Val	Thr	His	Gly	Thr	Asp	Thr	Leu		
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Gly	Pro	Met	Asn	Leu	Leu	Ser	Ser	Val	Thr	Leu	Ala	Ala	Ala	Ala	Ser		
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gcc	cct	gac	agt	ggc	atg	ctg	gga	aca	ttc	gtc	aac	gtt	cag	cca	gtg		976
Ala	Pro	Asp	Ser	Gly	Met	Leu	Gly	Thr	Phe	Val	Asn	Val	Gln	Pro	Val		
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Phe	Phe	Tyr	Pro	Pro	Ser	Arg	Pro	Leu	Gly	His	Arg	His	Phe	Asp	Leu		
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Ile	Val	Ser	Arg	Arg	Pro	Glu	Gly	Gly	Phe	Val	Gly	Pro	Cys	Glu	Ala		
			400				405						410				
gga	atc	ggc	gcg	ggc	ttt	ttg	aat	ccg	caa	aag	gcg	agg	atc	cag	ctc		1360
Gly	Ile	Gly	Ala	Gly	Phe	Leu	Asn	Pro	Gln	Lys	Ala	Arg	Ile	Gln	Leu		
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Ile Ser Thr Thr Ser Gly Phe Asn Cys Phe Asn Pro Asn Leu Pro Asn
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Val Thr Ile Tyr Ala Thr Gly Gly Thr Ile Ala Gly Ser Ala Ser Ser
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Ala Asp Gln Thr Thr Gly Tyr Arg Ser Ala Ala Leu Gly Val Asp Ser
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Leu Ile Asp Ala Val Pro Gln Leu Cys Asn Val Ala Asn Val Arg Gly
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Arg Thr Leu Ala Lys Gln Ile Gln Asn Asp Leu Asp Ser Pro Phe Thr
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Gln Gly Ala Val Val Thr His Gly Thr Asp Thr Leu Asp Glu Ser Ala
 195 200 205

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 Gly Ser Met Arg Pro Ala Thr Ala Ile Ser Ala Asp Gly Pro Met Asn
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 Thr Lys Val Asn Ala Asn His Leu Asp Ala Phe Gln Ala Pro Asp Ser
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 Ser Ala Leu Pro Gln Val Asp Val Leu Tyr Ala Tyr Gln Glu Leu Ser
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cta gct gct acg agt tat gcc tct ccc atc att cat tcc cgg gcc tcc      99
Leu Ala Ala Thr Ser Tyr Ala Ser Pro Ile Ile His Ser Arg Ala Ser
      15              20              25

aac acg tcc tat acc aac tct aat ggg ctg aaa ttt aac cat ttc gac      147
Asn Thr Ser Tyr Thr Asn Ser Asn Gly Leu Lys Phe Asn His Phe Asp
      30              35              40

gct tct ctt cca aat gtg act ttg ctg gca act ggt gga act att gcc      195
Ala Ser Leu Pro Asn Val Thr Leu Leu Ala Thr Gly Gly Thr Ile Ala
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ggt aca agc gat gac aag act gct acg gca gga tat gaa tcc ggg gct      243
Gly Thr Ser Asp Asp Lys Thr Ala Thr Ala Gly Tyr Glu Ser Gly Ala
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tta ggg ata aat aag att ctt tcc ggc atc cca gaa gtt tat gac att      291
Leu Gly Ile Asn Lys Ile Leu Ser Gly Ile Pro Glu Val Tyr Asp Ile
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gcc aac gtc aat gcg gta cag ttt gac aat gtc aac agc ggc gat gtc      339
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tct yca tct ctc tta ctg aac atg aca cat acc ctt caa aag acc gtt      387
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tgt gat gac cct acg ata tct ggc gcc gtc atc acc cat ggc acc gat      435
Cys Asp Asp Pro Thr Ile Ser Gly Ala Val Ile Thr His Gly Thr Asp
      125              130              135              140

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Thr Leu Glu Glu Ser Ala Phe Phe Ile Asp Ala Thr Val Asn Cys Gly
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aag ccg att gtg ttc gtt ggc tca atg cga cct tcc acc gca atc tct      531
Lys Pro Ile Val Phe Val Gly Ser Met Arg Pro Ser Thr Ala Ile Ser
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gcc gat ggc cct atg aat ttg ctc cag gga gtg act gtg gcc gct gac      579
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gtc tct gct ttc ttc gct aca aag aca aat gcg aat aca atg gac act      675
Val Ser Ala Phe Phe Ala Thr Lys Thr Asn Ala Asn Thr Met Asp Thr
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ttc aag gct tat gaa caa ggc agt ctt ggc atg att gtt tca aac aag      723
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cat ctt gac gac gtg gat gcg atc ccc cgt gtg gat att ctc tac gct      819
His Leu Asp Asp Val Asp Ala Ile Pro Arg Val Asp Ile Leu Tyr Ala
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tac gag gac atg cat agc gac tcc ctt cac agt gct atc aaa aat gga      867
Tyr Glu Asp Met His Ser Asp Ser Leu His Ser Ala Ile Lys Asn Gly
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Ala Lys Gly Ile Val Val Ala Gly Glu Gly Ala Gly Ile Ser Thr
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aag ttt gag gat att cga act atc ttc gga aaa gct act gtt gcc      1152
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Asn Val Thr Leu Leu Ala Thr Gly Gly Thr Ile Ala Gly Thr Ser Asp
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Asp Lys Thr Ala Thr Ala Gly Tyr Glu Ser Gly Ala Leu Gly Ile Asn
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 Val Asp Ala Ile Pro Arg Val Asp Ile Leu Tyr Ala Tyr Glu Asp Met
 260 265 270
 His Ser Asp Ser Leu His Ser Ala Ile Lys Asn Gly Ala Lys Gly Ile
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 305 310 315 320
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Ser Ala Lys Thr Arg Ile Ala Ser Gly Met Tyr Asn Pro Gln Gln Ala
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 130 135 140

Ala Gly Ala Met Arg Pro Ala Thr Ala Thr Ser Ala Asp Gly Pro Met
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Asn Leu Tyr Gln Ala Val Ser Ile Ala Ala Ser Glu Lys Ser Leu Gly
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Arg Gly Thr Met Ile Thr Leu Asn Asp Arg Ile Ala Ser Gly Phe Trp
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Thr Thr Lys Met Asn Ala Asn Ser Leu Asp Thr Phe Arg Ala Asp Glu
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Gln Gly Tyr Leu Gly Tyr Phe Ser Asn Asp Asp Val Glu Phe Tyr Tyr
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Pro Pro Val Lys Pro Asn Gly Trp Gln Phe Phe Asp Ile Ser Asn Leu
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Gly Leu Asn Pro Glu Leu Ile Val Lys Ala Val Lys Asp Leu Gly Ala
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28

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK 03/00684

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A23L1/03 A21D8/04 A23L1/217 A23L1/105 C12N9/82
C12N15/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A23L A21D C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/28729 A (NOVONORDISK AS ; SI JOAN QI (DK)) 22 December 1994 (1994-12-22) claims 1,12,13,16,17 page 9, paragraph 1 -----	1,4-6
X	WO 94/28728 A (NOVONORDISK AS ; SI JOAN QI (DK)) 22 December 1994 (1994-12-22) claims 1,5,11 page 8, paragraph 1 -----	1,4-6
X	US 2002/004085 A1 (OLSEN HANS SEJR ET AL) 10 January 2002 (2002-01-10) the whole document ----- -/--	1,6-8

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

23 January 2004

Date of mailing of the international search report

09/02/2004

Name and mailing address of the ISA

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Vuillamy, V

INTERNATIONAL SEARCH REPORT

Internat plication No

PCT/DK 03/00684

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/00029 A (NOVONORDISK AS ;WAGNER PETER (DK); NIELSEN PER MUNK (DK)) 8 January 1998 (1998-01-08) page 9, line 22 - line 31 page 6, line 29 - page 7, line 9 -----	1-5,7,9
X	WO 02/30207 A (BUDOLFSEN GITTE ;NOVOZYMES AS (DK); CHRISTIANSEN LUISE (DK)) 18 April 2002 (2002-04-18) claims; example 1 -----	1,2,6
X	US 6 039 982 A (SI JOAN QI ET AL) 21 March 2000 (2000-03-21) column 4, line 24 - line 39 column 6, paragraph 2 - paragraph '0003! claims -----	1,2,4-6
X	DATABASE WPI Section Ch, Week 199815 Derwent Publications Ltd., London, GB; Class D11, AN 1998-162469 XP002235162 & JP 10 028516 A (KAO CORP) 3 February 1998 (1998-02-03) abstract -----	1,2,4-6
X	PATENT ABSTRACTS OF JAPAN vol. 1997, no. 05, 30 May 1997 (1997-05-30) & JP 09 009862 A (CALPIS FOOD IND CO LTD:THE;AJINOMOTO CO INC), 14 January 1997 (1997-01-14) abstract -----	1
A	"Brief Communications" NATURE, vol. 419, 3 October 2002 (2002-10-03), pages 448-449, XP002235161 USA cited in the application the whole document -----	1
A	BIEKMAN E S A: "TOEPASSING VAN ENZYMEN BIJ DE VERWERKING VAN AARDAPPELEN TOT CONSUMPTIEPRODUCTEN" VOEDINGSMIDDELEN TECHNOLOGIE, NOORDERVLIET B.V. ZEIST, NL, vol. 22, no. 20, 12 October 1989 (1989-10-12), pages 51-53, XP000069625 ISSN: 0042-7934 the whole document -----	1,4,5,7, 8

-/--

INTERNATIONAL SEARCH REPORT

Internatl Application No

PCT/DK 03/00684

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/39828 A (DANISCO ; SOE JOERN BORCH (DK); PETERSEN LARS WEXOE (US)) 23 May 2002 (2002-05-23) claims; example 11 -----	1
A	K.W. KIM: "Asparaginase II of Saccharomyces cerevisiae" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 24, 1988, pages 11948-11953, XP002266820 USA cited in the application the whole document -----	3

INTERNATIONAL SEARCH REPORT

Inter application No.
PCT/DK 03/00684

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/UK 03/00684

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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JP 09009862	A	14-01-1997	NONE	
WO 0239828	A	23-05-2002	AU 1942202 A CA 2427914 A1 EP 1341422 A2 WO 0239828 A2 US 2002114864 A1	27-05-2002 23-05-2002 10-09-2003 23-05-2002 22-08-2002